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Transduction-like Gene Transfer in the Methanogen *Methanococcus voltae*

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ABSTRACT

Strain PS of *Methanococcus voltae* (a methanogenic, anaerobic archaebacterium) was shown to generate spontaneously 4.4 kbp chromosomal DNA fragments, that are fully protected from DNase and, upon contact with a cell, transform it genetically. This activity, here called VTA (*voltae* transfer agent), affects all markers tested: three different auxotrophies (histidine, purine and cobalamin) and resistance to BES (2-bromoethanesulfonate, an inhibitor of methanogenesis). VTA was most effectively prepared by culture filtration. This process disrupted a fraction of the *M. voltae* cells (which only have an S-layer covering their cytoplasmic membrane). VTA was rapidly inactivated upon storage. VTA particles were present in cultures at concentrations of approximately 2 per cell. Gene transfer activity varied from a minimum of 2×10^{-5} (BES resistance) to a maximum of 10^{-3} (histidine independence) per donor cell. Very little VTA was found free in culture supernatants. The phenomenon is functionally similar to generalized transduction, but there is no evidence, for the time being, of intrinsically viral (i.e. containing a complete viral genome) particles. Consideration of VTA size makes their existence unlikely. If they exist, they must be relatively few in number; perhaps they differ from VTA particles in size and other properties, and thus escaped detection. Digestion of VTA DNA with *Alu*I restriction enzyme suggests that it is a random sample of the bacterial DNA, except for a 0.9 kbp sequence which is amplified relative to the rest of the bacterial chromosome. A VTA-size DNA fraction was demonstrated in a few other isolates of *M. voltae*.

INTRODUCTION

Much progress has been made in the last twenty years in the understanding of the biochemistry of bacterial methanogenesis. The recognition of methanogens as archaebacteria (16) made them also very interesting from a broader biological perspective. A recent comprehensive review (44) covers the molecular biology of methanogens. Unfortunately, their slow growth and strict anaerobiosis have tended to discourage those experimental approaches that require much culture manipulation. Among methanogens, *Methanobacterium* and *Methanococcus* are the two best studied genera. They differ in several respects, the most striking being the absence of a true cell wall in *Methanococcus*, which, like numerous other archaebacteria, has nothing but a thin S-layer covering the plasma membrane. Concerning *Methanococcus voltae*, the object of the present work, a comprehensive review to 1989 is available (29). Later developments may be traced from recent publications (2, 27,32) (see also ref. 8 and 58 for the related *M. maripaludis*). Strain PS of *M. voltae* has been known to undergo low frequency, natural DNA transformation (6). Another, more efficient natural gene transfer mechanism, discovered a few years back (4, 5), is studied in this paper. The agent responsible for this activity will be referred to as VTA (*voltae* transfer agent). The phenomenon may be described as a generalized transduction, where however the bacteriophage component has not been detected and is probably absent or defective or different in size from the transducing particles or, in any case, present in very small amounts.

A variety of defective lysogenic systems have been described among eubacteria. Of particular interest in connection with the VTA case are those systems that accomplish transduction in the absence of "true", i.e. lytically self-reproducing, viral particles, as first found in *Rhodopseudomonas capsulata* (now *Rhodobacter*) (37, 66), and later in *Desulfovibrio desulfuricans* (43), *Serpulina hyodysenteriae* (24), and possibly *Myxococcus* (53). The *Rhodobacter* case, for which the new term "capsduction" has been introduced, is particularly interesting because of the small size of the bacterial DNA fragments transferred, almost equal to that observed here for VTA. Also, it has recently found application as a tool in the analysis of the *Rhodobacter* genome sequence (33). A more thorough comparison of VTA with the above mentioned systems will be found in the Discussion.

The only other case of generalized transduction among archaebacteria has been reported for *Methanobacterium thermoautotrophicum* (39) and involves a typical, medium size bacteriophage.

MATERIALS AND METHODS

Note. For anaerobic techniques, storage of bacterial strains, media composition, bacterial DNA extraction, and OD measurements, (6) should be consulted. Whenever a method evolved over time in the course of the work, only the most satisfactory version is described. 2-bromoethanesulfonate (Na salt) is abbreviated as BES.

Bacterial strains. Most were derivatives of strain PS of *Methanococcus voltae* and are listed in Table 1. *M. voltae* strains C1, C2, C4 and A3 (62, 64) were obtained from W. B. Whitman, University of Georgia, Athens; *M. vannielii* (52) was from J. N. Reeve, Ohio State University, Columbus.

Bacteriophages ΦX174 (50) and P2 *lg del1 del2* (7) were used as markers in sedimentation. For mixtures of the two phages, *Escherichia coli* strain C-1172, a P2 resistant mutant of C-1055 (63), was used to detect ΦX174, and *E. coli* strain K-221 (23) to detect P2.

Cultures, 5 ml in Bellco anaerobic culture tubes or 25 ml in 125 ml serum bottles, were grown in WM medium plus needed supplements at 30 to 33°C as described previously (6). They were used for experiments at an OD_{600} generally between 0.3 and 0.7. In calculations, a ratio of 3.5×10^7 total bacteria per ml for an OD_{600} of 0.1 was assumed. For the strains more frequently used, fully grown cultures were stored at room temperature in the anaerobic hood for several weeks, repressurized now and then with H_2-CO_2 to delay lysis (6), and used as inocula (diluting 1:30 to 1:300) for new cultures. Stock cultures of each strain were stored frozen and retrieved when needed as earlier described (6). Viable, easily accessible inocula have been successfully maintained by this method for longer than twelve years.

"Tumbler" cultures (265 ml in 500 ml serum bottles) were grown at 18 psi from a large (1/20 of volume) inoculum of exponentially growing bacteria, and were used at OD_{600} between 0.1 and 0.4. To avoid foaming with minimum stress to the cells, gas exchange was facilitated by having the bottles lying down and gently rolling back and forth, hitting the walls of a container about 4 times per minute ("tumbling").

Media. Medium WM (6, 61) was generally used, but without resazurine. L-histidine (final 0.5 mM), hypoxanthine (final 0.2 mM) and cyanocobalamin (final 100 nM) were added at inoculation as needed for growth of strains carrying the mutations *his*, *pur* or *cbl* (63). BES was added for some selections, but BES resistant strains were generally grown without it. For frozen storage of cultures and their recovery, and on occasion to facilitate growth from old inocula or in the 250 ml, "tumbler" cultures, the medium was supplemented with 1/20 to 1/200 volume of a concentrated solution of Difco Casamino Acids, Yeast Extract and L-tryptophan (10., 5., and 0.1 % w/v, respectively).

Viable counts were made by the thin layer, soft agar, *pour plate technique* (6), which does not require preparing plates ahead of time. The agar concentration was critical: satisfactory results were obtained with 0.5% Difco Noble agar as with 0.34% Fisher Scientific laboratory grade agar (6). Before inoculation, each melted soft agar tube (already containing the added sulfide solution and any other needed supplements) was removed from the 45 to 48°C heating block so it could cool down for 3 to 4 minutes. The plates were incubated for 10 to 12 days at 30 to 33°C. At variance from (6), no $CaCl_2$ was added to the canister. Also, instead of adding H_2S gas, an open container with a few ml of a 20% Na_2S solution was placed at the bottom of the canister (9). Colony size was strongly affected by crowding: when in high numbers, colonies were quite small and, except for a few spreaders, did not merge with each other. For some crowded plates, colony counts were made under the microscope at an appropriate magnification for a number of fields randomly chosen on the plate, then averaged and multiplied by the known plate to field area ratio.

Resistance to BES. *Methanococcus voltae* PS is sensitive to BES: a stationary phase inoculum grew with a 1 day lag in liquid culture with 1 µM BES; at 10 µM and 100 µM the growth rate was reduced 4- and 8-fold respectively; there was no growth at 1 mM BES. On plating in agar with 1 mM BES the efficiency of colony formation was usually well below 10^{-5}.

The BES resistant strain PS-6 grew normally in liquid culture at 1 mM BES and with a small delay at 2 mM BES. It did not grow at 10 mM BES. In agar, the efficiency of colony formation by stationary phase inocula was only slightly affected at 1 mM BES, but greatly reduced ($<10^{-3}$) at concentrations higher than 5 mM BES.

A high resistance strain (PS-13) was isolated as follows. Several 0.5 ml aliquots of a PS-6 culture, fully grown in the presence of 3 mM BES, were UV irradiated (6) (avoiding photoreactivation) with a dose expected to inactivate 96 to 99% of the cells, then added to 5 ml of WM with the required supplements and 2 mM BES, and incubated to turbidity. From each culture 1 ml was plated with 7.5 or 9 mM BES. Very variable numbers of colonies were obtained. In some case the colonies were obviously heterogeneous in morphology, as though mutants were present. Single colonies were picked and tested for growth in liquid culture with 9 mM BES. PS-13 is one of such isolates that, differently from its parent strain, grew satisfactorily in 9 mM BES and formed colonies with a thin halo of less compact growth. It was reisolated in the absence of BES and retested. It grew well in liquid culture with 10 mM BES, although with a 1 to 2 days lag; it also grew, although more slowly, in 15 mM BES. However, when the resistance to BES of PS-13 was compared to that of the parent strain *by plating* for colonies in BES containing agar (using stationary phase inocula), no convincing difference in resistance levels was noted. This has not been investigated further.

VTA assays were made basically like viable count assays. In the earlier experiments, recipient bacteria and filtrate samples were introduced directly into the melted soft agar tube using disposable syringes and Becton-Dickinson, gauge 22 needles, and measuring volumes in drops (holding the syringe vertically gives approximately 14 µl per drop). Later (this is now the preferred method) a measured volume of donor filtrate (between 50 and 200 μl; diluted if necessary) was prepared in a 1.5 ml Eppendorf disposable vial, kept on ice, in air, and transferred to the anaerobic hood minutes before plating. A drop of a fresh culture of recipient bacteria (OD_{600} between 0.4 and 0.7) was delivered by syringe to the (10 ml) melted soft agar tube through the stopper. Then, immediately, using a new needle and syringe, about 0.5 ml were removed from the agar tube and gently blown into the sample in the vial; the whole was sucked up again into the syringe and delivered through the stopper to the melted agar tube. To completely deliver the sample, the syringe was rinsed out into the tube by filling and emptying it once more. The stopper was removed with forceps and the agar poured into a Petri dish. Recipient cultures, kept at room temperature and repressurized with H_2-CO_2, were sometimes used again in new platings: after a few days, however, the efficiency of gene transfer decreased significantly.

Sterile filtrates. Fresh cultures were filtered by hand pressure using either Gelman Science Acrodisc, 0.2 μm pore size, 26 mm diameter, polysulphone filter cartridges, or Schleicher & Schuell UNIFLO, 0.2 μm pore size, cellulose acetate filter cartridges; they were equally satisfactory. Filters became clogged after passage of 5 to 15 ml of culture, depending on the bacterial concentration. Some other types of commercial membrane filters, also of 0.2 μm porosity, did not give as high yields of VTA as the two mentioned above. Filters of 0.45 μm pore size clarified the cultures more rapidly, but occasionally let through a small fraction (below any visible turbidity) of *Methanococcus* cells, and were then avoided. The filters were not usually washed before use. The error in filtrate absorbance readings due to material leached from the membranes was negligible.

VTA stability measurements. VTA preparations were usually titrated the day they were made. Later assays supplied estimates of VTA stability upon storage and/or dilution. VTA inactivation was expressed as the number of lethal hits, $H = -\ln S$, where S is the VTA survival ratio (titer at time t_2 to titer at time t_1). More specifically, H_0 is the estimate based on the first titration after beginning of storage (or after dilution into a different suspension medium) compared with the initial titer, whereas $H_{mo} = -(365/12) \ln S/d$ (where $d = t_2 - t_1$ days of storage) is the rate (lethal hits per month) based on any two successive titrations, past the initial one. When more than one H_{mo} estimate was available for a given preparation, an average $H_{mo}(n)$ of the n estimates was calculated. Alternatively, $H_{mo}(n)$ was calculated as the linear regression coefficient of the logarithm of the VTA titer *vs* days of storage.

VTA preparation and concentration. The simplest method was by direct filtration of cultures. Since most of VTA in a culture was found to be associated with the cells (see Results), another simple method consisted in sedimenting the bacteria and filtering after resuspending them in a buffer. At earlier stages of the work, however, numerous attempts were made to separate VTA from the bacteria by what was presumed would be desorption, as in the following example. Donor bacteria were grown (e.g. 2 bottles, 53 ml total volume) to OD_{600} 0.3 to 0.5 (about 3 days incubation from a 1:50 inoculum). They were transferred in the hood to polypropylene centrifuge tubes and spun anaerobically for 5 min at 10,000 rpm (Sorvall S34 rotor) in the cold. The tubes were opened in the hood and the supernatants discarded, draining well. The pellets were gently resuspended using a "squeezette" (soft plastic transfer pipette) and pooled in total 10 ml cold 0.3 M NaCl with 5 mM Na citrate. The suspension was spun again as above. The new supernatant was collected and filtered (F_1). The new pellet was resuspended and centrifuged again as before. The next supernatant was collected and filtered (F_2). This process could be repeated. In the end, the pellet was resuspended as before and filtered (F_p). For this last filtration, because of the high bacterial concentration, more than one filter often had to be used. BSA (final concentration 1 or 2 mg/ml) was added to the filtrates and they were stored on ice. Of the total VTA recovered about half was in the last filtrate (see Fig. 3). The total active VTA yield, measured as *his*$^+$ transfer, varied between 2×10^4 and 3×10^5 per ml of culture.

Concentration and purification were problematic due to VTA's inherent instability and to the laboriousness of filtration, especially at high bacterial concentrations. Direct sedimentation of VTA by centrifugation gave large activity losses (due to pressure effects (18)? sticking to the tube walls? irreversible aggregation?), except when a sucrose cushion was used. Centrifuge aided concentration in Centricon 500 filters (Amicon, Inc.) was slow (about 5 ml per hr for filtrates of culture supernatants) and gave low recovery (e.g. 20%) of activity. In preliminary experiments VTA was precipitated by PEG (5 to 30% w/w), but only 5% of the activity was recovered from the sediment, even though stability upon storage at such PEG concentrations was about average for VTA suspensions in saline (see Table 3). Of other procedures tested, two are described below. The first, (a), aimed at concentrating VTA that was (as much as possible, see under Results) set free from the cells prior to filtration. The second procedure, (b), is the more satisfactory for rapid concentration of VTA without much activity loss, and could be used as the starting point for purification of the material.

(a) Pooled cultures (210 ml, OD_{600} 0.3) were centrifuged (Sorvall GSA rotor, 7,000 rpm, 10 min) anaerobically. The pellet was resuspended in 2 ml 0.3 M NaCl with

$MgCl_2$ (5 mM) and DNase (5 μg / ml). Soon after, buffer (0.3 M NaCl, 20 mM Tris, 1 mM EGTA, pH 7.6) was added to reach 20 ml, and the suspension was spun again (Sorvall SS-34 rotor, 10,000 rpm, 5 min). The supernatant was collected; the pellet was resuspended in 20 ml buffer, again centrifuged, and the supernatant was pooled with the first. The new pellet, resuspended in 2 ml 0.3 M NaCl, 5 mM $MgCl_2$, was again treated briefly with DNase, then buffer was added to 20 ml, and the suspension centrifuged. The three pooled supernatants were filtered and then spun (Spinco SW28 rotor, 18,000 rpm, 5 hours, 8°C) on a cushion of 1 ml 66 % w/w sucrose. The bottom 1.5 ml from each tube was collected after removing the supernatant from the top, pooled and dialyzed against buffer (without EGTA) to remove the sucrose. The dialysis bag was then placed in a bed of dry PEG (6,000 MW) for 30 min to reduce the volume. The suspension was again treated with DNase (added to obtain 6 μg per ml, with $MgCl_2$ to 10 mM) for 1 hour, then with RNase (added to 150 μg per ml) for 30 min, then spun (Spinco SW50.1 rotor, 32,000 rpm, 8°C, 4 hours) on a 1 ml cushion of sucrose (as above). Fractions were collected dropwise and their refraction index was measured. Based on prior calibration, those expected to contain most of the band of VTA activity, were pooled and dialyzed against 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.8. This procedure reduced the original culture volume 420 ×. The activity recovered was only a few percent of the original, however.

(b) PEG-bag method. Fresh cultures at about 10^8/ml total count were spun (Sorvall GSA rotor, 7,000 rpm, 10 min, 5°C) anaerobically. The well drained pellets were resuspended in buffer (0.3 M NaCl, 20 mM Tris, 1 mM EGTA, pH 7.5) at about 2/3 of the original volume, and filtered. The filtrate was transferred to dialysis tubing (flat width

25 mm; prepared by heating at 80°C for 30 min in 10 mM EDTA, at neutral pH, then rinsed with buffer, then left a few hours before use, filled with a 10 mg/ml BSA solution in buffer), which was then packed all around with PEG (polyethylene glycol 6,000) crystals, wrapped in plastic film and stored in refrigerator (9 to 10 hours) till most of the fluid had been absorbed out by the PEG. The tubing was then very rapidly rinsed with running water on the outside to remove PEG stuck to it, then opened at one end and laid flat on a clean sheet of "Parafilm" or other water repellent surface. The contents were squeezed out onto the surface by rolling a thick glass rod from the sealed end of the tubing to the other end, and collected. A very small volume of water was added to the tubing, trying to mix it as well as possible with whatever was adsorbed to the inside surface of the tubing, and then rolled out as above and collected. This operation was repeated once more. The VTA concentrate obtained was rich in BSA and salt, but showed a 40 to 100% recovery of VTA activity with a 100 × volume reduction. It was stored frozen (see Table 3).

"Short-cut" preparation of VTA DNA. Bacteria from two "tumbler" cultures were pelleted anaerobically at 9,000 g (Sorvall GSA rotor, 7,000 rpm, 10 minutes). All supernatant fluid was removed. The bacteria were resuspended in 1 ml 0.3 M NaCl, 20 mM Tris, pH 7.7, with BSA (1 mg/ml), pooled, then lysed by the addition of 200 ml of ice-cold distilled water, mixing rapidly. Small volumes of NaCl, $MgCl_2$ and DNase (Sigma DN-25) solutions were added to obtain final concentrations of 50 mM, 1 mM and 2 µg/ml, respectively, and the mixture was warmed up to room temperature. After 20 minutes, RNase was added to a final concentration of 25 µg/ml. Forty minutes later, EDTA was added to a 2 mM final concentration. VTA and bacterial debris were immediately pelleted at 44,000 g (Spinco rotor SW28, 18,000 rpm, 4:30 hours, 8°C), resuspended in 3 to 4 ml STE2 buffer (100 mM NaCl, 10 mM Tris, 2 mM EDTA, pH 8.0), and extracted with phenol (with hydroxyquinoline and β-mercaptoethanol) through chloroform (45) and concentrated by precipitation with cold ethanol (45) in a thick wall, glass centrifuge tube. Mussels glycogen was added at 20 μg/ml before the ethanol, as a carrier (19, 56). After a day at -20°C, the ethanol mix was centrifuged (Sorvall SS-34 rotor, 10,000 rpm, 1 hr, near 0°C) and the pellet was dried and suspended in the smallest possible volume of STE (45). No residual DNase activity was detectable in the final preparation. With strains PS-1 and PS-13 total yields of about 1 μg VTA DNA could be reproducibly obtained. VTA DNA was further purified and concentrated by cutting out bands in agarose gel after electrophoresis and extracting the DNA by the QIAGEN method.

Other nucleic acid methods were standard (11, 45). Gel electrophoresis, was usually performed in horizontal submerged 0.8% agarose gel slabs (25 ml, 95 × 65 mm) in buffer (40 mM Tris, 5 mM Na acetate, 1 mM EDTA, pH 7.4), at 100 V (8 V/cm gradient) for 80 min. The gels of Fig. 6, B and of Fig. 7 had however 1% agarose, and were run at 50 V for 200 min. The gels were stained in 1 μg/ml ethidium bromide for 20 min, and destained in 1 mM $MgSO_4$ for 40 min before being photographed. Restriction enzymes were used as recommended by the commercial enzyme suppliers. DNase refers to DNase I and RNase to RNase A throughout.

RESULTS

VTA activity. When the histidine-requiring PS-3 mutant strain of *Methanococcus voltae* was grown in mixed culture with the purine-requiring PS-6 strain in fully supplemented medium, large numbers (e.g. 10^4 per ml) of His^+Pur^+ colonies were obtained upon plating in the appropriate selective medium. The frequency of prototrophs in pure cultures of either strain was extremely small (<50/ml). Large numbers of prototrophs were found also in mixed cultures to which DNase had been added at inoculation: such prototrophs could then hardly result from the low level of DNA-mediated, natural transformation that was earlier demonstrated for *M. voltae* PS (6). These observations suggested the presence of some other kind of genetic recombination process and were then extended as follows.

a) High numbers of prototrophs were routinely obtained by plating bacteria of the one strain with a bacteriologically sterile filtrate of the other strain (Table 2). Gene transfer was thus mediated by a filterable, subcellular agent, to be called here VTA.

b) For any given filtrate and recipient culture, the number of prototrophs obtained was linearly related to the volume of filtrate plated over a wide range. Lower efficiencies of transfer (saturation of receptors? cell killing?) were observed (data not shown) when the recipient and an excess of VTA were mixed and incubated before the selective plating.

c) The number of prototrophs varied with the concentration of recipient in the agar (Fig. 1). At very high bacterial concentrations no transformant colonies were obtained. The residual growth of the auxotrophs present in excess apparently interfered with the growth of the few transformed cells: in reconstruction experiments, cells of prototrophic strain PS-12 did not form colonies when mixed with such an excess of PS-3 auxotrophs in the absence of histidine, but they did if the PS-3 bacteria had been first heat inactivated. As the bacterial concentration decreased (Fig. 1), the number of transformants also decreased, as one might expect for a time cut off (irreversible incapacitation of the auxotrophic, recipient bacteria and/or spontaneous inactivation of VTA *vs.* diffusion and adsorption rates). It was at first surprising that transformed colonies could be obtained at recipient concentrations in the agar as low as 10^4/ml. Calculations based on Schlesinger's adsorption equation (46) showed however that even at such a low bacterial concentration most VTA particles would be adsorbed in less than 48 hours if the adsorption constant were in the range of values established for bacteriophages. For the same marker (*his*$^+$) selection, the optimal input of recipient bacteria varied among cultures between 10^6 and 10^7 per plate.

d) All three auxotrophic mutations available, *his*, *pur*, and *cbl*, were transformed to wild type by appropriate filtrates (Table 2). In the case of *cbl*, results were at first ambiguous (Table 2, compare E10, E12 with E9, E11), presumably because the amount of cyanocobalamin carried over with the inoculum was sufficient (see Fig.6 in ref. 6) to produce substantial amounts of residual growth, accompanied by some back-mutation, in the selection plates. (For the same reason the colonies in line E9 of Table 2 ought to be interpreted as transformed only to *pur*$^+$). Transfer of *cbl*$^+$ was confirmed with a more critical experiment (Table 2, J1, J2, J3) where the capacity for residual growth of the *clb*$^+$ cells was reduced by using recipient cultures grown at lower concentrations of cyanocobalamin.

e) The level of VTA activity for the individual filtrates covered in Table 2 was very variable, from 1.4×10^3 to 2.2×10^5 per ml of filtrate, with overall average 3.9×10^4. The averages for the transfer of *cbl*$^+$, *his*$^+$, and *pur*$^+$ were, respectively, 3.1×10^4 (n = 2), 6.4×10^4 (n = 7), and 2.0×10^4 (n = 8) per ml of culture. In first approximation, assuming that 3.5×10^7 bacteria per ml correspond to an optical density of 0.1 (6) and that the average

A3, further work ought to consider the possibility that this plasmid, or parts of it, be the repository of the genes necessary for VTA production.

VTA is rather unstable under all conditions tested to-date. From Table 3, the average H_{mo} (defined in Materials and Methods) at ordinary refrigerator temperatures is 1.8. This seems to be of the same order of magnitude as for the least stable *Escherichia coli* bacteriophages, e.g. Mu. The comparable H_{mo} for a moderately stable *E. coli* bacteriophage, wild type P2, would be 0.04 (unpublished data).

The mechanism of VTA production is very puzzling. Most of the VTA in a culture is found somehow adsorbed to, or complexed with, the cells (or with large, easily sedimentable cell debris), and much of it is efficiently set free only upon filtration, presumably as a result of shear and/or cell disruption. This situation might reflect either the process by which VTA is produced or what happens to VTA after it is produced. In bacteria with an external S-layer, bacteriophages specifically adsorb to it (14, 21, 26, 34), but the reversibility of this step has not been studied. Perhaps injection of the DNA (an irreversible step) occurs only when the S-layer is locally in proper contact with the plasma membrane. Such a situation would be compatible with the recovery of VTA activity upon desorption or filtration. Observations reported elsewhere (15) make it very probable that VTA consists of tailed, bacteriophage-like particles. The buoyant density of VTA in CsCl gradients is exceptionally low compared with literature data for bacteriophage particles of similar structure. This may be explained if the particles should happen to be easily, but still reversibly, entrapped by cell surface structures. This hypothesis would also explain the stubborn association of the bulk of VTA with the cells. More speculatively, *M. voltae* might be capable of generating membrane vesicles, for example like the ones that have optical density of the donor cultures used in Table 2 was 0.6, the average VTA activity for any given nutritional marker in a donor culture would be about 0.02% per bacterium. For the *his*$^+$ transfer, this value could be increased 5× to 10× in later, better controlled experiments, where the bacterial concentration of the donor culture at filtration was kept between 1 and 2×10^8 per ml.

f) The same strain could be used as donor or recipient, provided an appropriate marker could be selected. All combinations of derivatives of *M. voltae* PS tested gave similar results (Table 2). Thus VTA is present in all derivatives of strain PS. On the other hand, filtrates of *M. voltae* strains A3 (64), C1, C2 and C4 (62) did not seem to be able to transfer *his*$^+$ to PS-3 or *pur*$^+$ to PS-13. Likewise, no transfer of *pur*$^+$ to PS-6 could be obtained with a filtrate of *M. vannielii*.

g) To show that VTA also acts on properties other than nutritional requirements, the transfer of resistance to BES was studied. Results were negative when selection for BES resistance was applied directly to sensitive cells mixed in agar with VTA from a *bes* strain. This is what one may expect if *bes* were a recessive mutation. When BES sensitive bacteria were plated as usual with filtrate from a BES resistant strain, allowed to grow for a while, and then challenged with BES, transfer of *bes* could indeed be measured (Fig.2). The frequency of transfer of *bes* was much lower than that of nutritional markers, however: for four VTA preparations from PS-2 it ranged from 0.3% to 1.9% of that of *his*$^+$.

h) VTA was inactivated by heating (Table 2, B3, E11). It was quite resistant to the manipulations with syringe and needle required in diluting and plating (Table 2, K1, K2).

i) Whereas *M. voltae* cells promptly lysed when diluted in distilled water (reviewed in ref. 62), VTA activity survived well such dilution (Table 2, G4).

j) VTA was not affected by DNase (Table 2, C4, C5). Likewise DNase added to the donor culture before filtration did not reduce VTA activity (and improved somewhat the rate of filtration for more dense cultures). RNase, at as high a concentration as 1 mg/ml, did not affect a filtrate's transfer activity.

k) VTA could also be demonstrated following removal of bacteria by centrifugation and filtration of the supernatant. The activity in this case was always much lower than if the whole culture was filtered (Table 2, A3, E5, E6, H3, I3). Apparently most of VTA was not free. Resuspension of the bacteria in saline appeared to set free some VTA activity prior to filtration (Table 2, I4) without visible cell lysis. On the other hand, interactions at the filter, presumably shearing effects, were clearly important: the VTA titer from cultures was always very low in the first ml or so of filtrate, rapidly rose to a maximum and then decreased when the filter became overloaded with bacteria and the flow rate was strongly reduced (Table 2, F1 through F3, and other experiments not shown). Calculations suggest that the free setting of VTA at the filter started roughly when the equivalent of a monolayer of bacteria was trapped on the filter surface. The difference in VTA activity between the first fraction and the rest of the filtrate was noted also when the filter had been extensively washed prior to use with the same medium in which the bacteria were suspended, in order to obviate possible ionic effects at the filter.

l) Attempts to induce increased VTA production by transiently exposing donor cultures to heat, UV light, or mitomycin C were unsuccessful. Autolysis following starvation for H_2 (6) did not seem to affect the VTA titer.

Mode of VTA liberation. The bulk of VTA is associated with the bacterial cells and very little is free in the culture supernatants (see above, under k). A larger fraction of VTA could be recovered in supernatants through "washing" (pelleting of cells in the centrifuge, resuspension in a buffer, new pelleting, filtration of the supernatant). Although salt solutions of diverse composition and concentrations were tried (0.15 to 1.5 M NaCl, with or without 15 to 60 mM Na citrate, 8 to 60 mM Tris, 0.5 to 5 mM EGTA, and pH between 6 and 8), there were no striking differences in the results: even with repeated washings, longer waiting times or higher temperatures after resuspension, or lower cell concentrations (to reduce possible readsorption of VTA), only rarely (e.g. Table 2, I4) more than half of the total VTA could be recovered by this procedure, the rest being recoverable only upon filtration of the bacteria themselves (Fig. 3). For 26.5 ml cultures of strains PS-2 or PS-13 at between 10^8 and 3×10^8 bacteria per ml, the above procedure gave a total *his*$^+$ VTA yield between 5×10^5 and 8×10^6 (average 3.4×10^6, n=17, or, roughly, 6×10^{-4} *his*$^+$ VTA per cell).

Filtration caused substantial bacterial lysis, as indicated by the amount of UV absorbing material detectable in the filtrate. For example, a culture of strain PS-1, grown to OD_{600} 0.32, washed and resuspended in 0.3 M NaCl, then filtered, gave A_{260} 0.16 and A_{280} 0.11 in the filtrate. The same washed culture, totally lysed by resuspension in distilled water, gave A_{260} 0.38 and A_{280} 0.24. One would conclude that nearly half of the bacterial proteins and nucleic acids were set free and not retained by the filter. For comparison, the A_{260} of a similarly prepared filtrate of an *Escherichia coli* C culture (grown aerobically in mineral medium plus glucose) was only a few percent of its original A_{600}, and no peak at

A_{260} was evident. Phenol extracts confirmed the presence of RNA and high molecular weight DNA in *M. voltae* filtrates (see Fig. 6, D)

Similarly, high absorbance in the UV range was noted in VTA preparations obtained by washing (as defined above). This suggests that some cell lysis may also occur as a result of the centrifugation and resuspension process. Indeed, the amounts of cell lysis (measured as solubilized, UV absorbing material) and of VTA activity recovered were roughly correlated within a given experiment (Fig. 4).

Other properties of VTA. Most of the experiments of Table 2 were done with freshly prepared culture filtrates. The VTA activity in such preparations, even under refrigeration, decayed quite rapidly. At first VTA was routinely stored under anaerobic conditions. It was soon realized however that the gain in stability as compared with storage in air, if any, was small. All preparative operations with VTA following filtration were since done without anaerobic precautions.

Longer time series of VTA titrations suffered from the variability in the efficiency of plating, which is difficult to control since a different recipient culture has to be used at each time. The data available for a number of VTA preparations were tentatively interpreted as showing a bimodal decay over time: a sharp drop in titer over the first day or two of storage, presumably due to an equilibration to the storage conditions, followed by a slower decay, which was assumed to follow a simple exponential function and was expressed as lethal hits per month, H_{mo} (see Material and Methods). Thus, for $H_{mo} = 1$, roughly two thirds of VTA activity would be lost during a one month storage period. Some examples are given in Table 3. The initial inactivation, H_0 (see Material and Methods), was not correlated with H_{mo}.

Conditions affecting VTA stability were not studied systematically. The still exploratory data available suggest that VTA (as *his*$^+$ transfer) stability is little affected by varying pH (between 6.7 and 7.9), NaCl concentration (between 0.15 M and 1.0 M) or by addition of $MgCl_2$, glycerol (5 to 20%) or chelating agents (5 mM to 75 mM Na citrate; 1 mM EDTA, 1 or 2 mM EGTA). VTA was rapidly inactivated in presence of high concentrations of CsCl or RbCl. It tolerated well rapid dilution, not storage, in H_2O. It could stand high concentrations of sucrose. BSA at ≥1 mg/ml, and possibly PEG at 100 mg/ml, improved somewhat VTA stability. In the presence of glycerol and/or high protein concentrations VTA fairly well withstood freezing and thawing; nevertheless, some decay continued to occur in the frozen state at the temperatures used (Table 3).

Attempts were made to establish the buoyant density of *his*$^+$ VTA in a CsCl density gradient. Because of its rapid inactivation, however, the density estimate obtained of 1.28 g/ml (Fig. 5), where the recovered VTA activity was 1% of input, may not be adequately representative. In an earlier CsCl equilibrium density run (data not shown) where the recovered activity was even lower ($<10^{-4}$), the few *his*$^+$ colonies obtained in the assays were distributed over a 1.34 to 1.37 density range. In the gradient of Fig. 5 there seems to be a small, possibly significant VTA peak at density 1.38 (at 1.6 ml cumulative fraction volume in the figure). This raises the possibility of two forms of VTA with different buoyant densities and different sensitivities to CsCl, one of them being rather tightly complexed to lighter material (see Discussion).

VTA DNA. Gel electrophoresis of total DNA preparations of *M. voltae* PS revealed, in addition to high molecular weight, chromosomal DNA, an extremely faint, rather broad secondary band, at about 4.4 kbp (as calculated for linear, double stranded

DNA) (Fig. 6, A). DNase eliminated both the chromosomal and the 4.4 kbp band. Both were unaffected by RNase. The band could be demonstrated in six DNA preparations (from strains PS-1, PS-6, PS-9, PS-12 and two subisolates) out of eight, made with slightly different methods over a number of years. The presence of such a band had not been noted in work from other laboratories (28, 49, 51, 62) that used *M. voltae* PS DNA for a variety of purposes. Presumably, the amount of DNA is affected by the preparation method and by the history of the culture. For two DNA preparations of known concentration, made from unwashed bacterial pellets, the amount of DNA in the 4.4 kbp band, very roughly estimated by comparison with the DNA size standards, amounted to less than 1% of the total DNA (0.1% and 0.4%, respectively, or, very roughly, one copy of 4.4 kbp DNA, on the average, per *M. voltae* chromosome (51) of 1,880 kbp).

The possibility that the 4.4 kbp DNA might be involved in the VTA activity was confirmed by the presence of DNA of the same size in partially purified VTA preparations. In a first experiment, the VTA particles in a culture filtrate were separated from soluble cell material by centrifugation over a sucrose cushion, then further concentrated by membrane filtration (always without addition of DNase or RNase) and extracted with phenol. The extract yielded a 4.4 kbp DNA band plus some material of lower molecular weight, presumably contaminant RNA (Fig. 6, B). A similar result (Fig. 6, C) was obtained with a different VTA concentration method (procedure (a) in Material and Methods), aiming at keeping the cells from lysing. In this case any free DNA had been eliminated by means of DNase. Both procedures were cumbersome and the recoveries of VTA activity, just before DNA extraction, were very low. For the isolation of VTA DNA a more efficient method was developed (see Materials and Methods, "Short-cut" preparation of VTA DNA), based on the resistance of VTA to DNase and on the assumption that VTA particles, if sedimented together with cell debris, might physically better survive pelleting (Fig. 6, E). Finally, enrichment in 4.4 kbp band material was also evident in extracts of concentrated (but far from pure) VTA preparations made by the PEG-bag method (Fig. 6, D).

At temperatures above 78° C (in 10 mM Tris, 0.6 mM EDTA, pH 8 at 20° C) VTA DNA was promptly denatured to a faster moving, broader band in gel electrophoresis (Fig. 6, F and G), indicating separation of the strands of linear (or nicked-circular), double stranded DNA molecules. (*M. voltae* DNA is known (62) to have very low GC content.)

Using the "short-cut" preparation method, DNA bands of 4.4 kbp could also be demonstrated in preparations from *M. voltae* strains C1, C2 and A3. In the case of A3 several slower, very faint bands were also present, which could be interpreted as forms of the plasmid pURB600, known to be present (64) in this strain, that were somehow protected from the DNase treatment.

No discrete restriction fragments were obtained from VTA DNA using several restriction endonucleases (*Ava*I, *Bam*HI, *Bgl*II, *Eco*RV, *Hpa*I, *Nde*I, *Pst*I, *Sal*I): the VTA DNA band was still present, in some cases less intense and with a faint smear on its low molecular weight side. (*Bam*HI and *Bgl*II reduced the electrophoretic mobility of the DNA band, presumably due to stable complex formation with the DNA). The VTA DNA band disappeared after digestion with *Alu*I, whose recognition site is only four nucleotides long, rather than six. Digestion of total *M. voltae* DNA with *Alu*I gave a complex pattern of bands (Fig. 7, A, lanes 1, 2, 3), all of which, with the exception of the slowest, *Alu*I band A, at 4.2 kbp, ought to be interpreted as collections of individual bands. That the largest bacterial DNA fragment, *Alu*I band A, is so near in size to VTA DNA may be at first surprising, but is not beyond rational expectations for a random distribution of *Alu*I restriction sites. Digestion of highly concentrated preparations of VTA DNA gave also a complex band pattern (Fig.7, A, B) which could be considered identical to that for total *M. voltae* DNA, except in two respects: *Alu*I band A was absent, and a new band (or collection of bands of nearly equal sizes), to be called *Alu*I band φ, was present at 920 bp (range 800 - 1,020 bp). The disappearance of the *Alu*I band A fragment is not unexpected, since, given its size, the probability of its inclusion, uncut, in the 4.4 kpb VTA DNA fragments would be quite low. Band φ was not affected by RNase (Fig.7, C). It could not be seen following double digestion of highly concentrated VTA DNA with *Alu*I and *Dra*I.

DISCUSSION

Based on the evidence presented, strain PS of *Methanococcus voltae* spontaneously generates DNase resistant particles, referred to here as VTA, that contain 4.4 kbp of DNA and are able to transfer to other cells anyone of four genetic markers tested. It is fair to assume that VTA will transfer almost any genetic marker on the *M. voltae* chromosome. It is shown elsewhere (15) that partially purified preparations of VTA contain bacteriophage-like (in the morphological sense) particles, with an isometric head and a tail, the head being of an appropriate size for harboring a 4.4 kbp molecule of DNA.

Is this a case of generalized transduction, where the appropriate host strain for bacteriophage plaque formation is missing? Circumstantial evidence made this unlikely and may be very roughly estimated as follows. VTA DNA corresponds in size to 1/423 of the 1,880 kbp (51) *M. voltae* chromosome. A culture of 2×10^8 bacteria per ml yields (by the "short-cut" preparation method) about 2 ng of VTA DNA per ml, equivalent to 4.2×10^8 VTA copies. Such a culture would give an activity (as *his*$^+$ transfer) of about 1.3×10^5 per ml (average of several similar experiments of the type exemplified in Fig. 3 for strains PS-2 and PS-13). The efficiency of transfer by VTA particles for *his*$^+$ may then be calculated as $1.3 \times 10^5/4.3 \times 10^8 = 0.031\%$, and, correcting for a sampling factor of 423 (see above), tentatively assumed to be constant over the bacterial chromosome, to R = 13%. The corresponding calculation for the transfer of another auxotrophic marker, *pur*$^+$, would give R = 4.1%. These figures do not take into account the possibility of inefficient integration (still undemonstrated in this system) of the transferred DNA fragments. If the efficiency of integration is assumed to be of the order of 1/10, as found for example in P22 (42), the above estimates of R would bracket 100%, a result clearly unlike the case of typical generalized transduction.

Second, the DNA molecules carried by VTA particles are quite small (4.4 kbp) when compared to those of any double stranded DNA bacteriophage (known range: 12 to 750 kbp). The genes necessary for specification of particle structure, control of replication, sizing of host DNA fragments, and their incorporation into the capsid could hardly fit into 4.4 kbp of DNA. (But note the arguable counterexample of polyoma and SV40 viruses, with 5 kbp circular DNA, where "pseudovirions" may contain host cell DNA fragments.)

Third, routine examination of VTA DNA after digestion with a variety of restriction enzymes suggested at first that it was nothing but a random sample of the bacterial DNA of *M. voltae*, and the possibility was considered that VTA might represent a new case of "capsduction", described (37, 60, 66) for strains of *Rhodopseudomonas capsulata* (now called *Rhodobacter*). These strains spontaneously produce particles (called GTA, for gene transfer agent) with 30 nm, isometric heads and short tails, containing 4.5 kbp of DNA and able to transduce any bacterial marker. Only bacterial DNA was found in the GTA particles (66). Their production would seem to be controlled and specified by bacterial genes, without any differential DNA replication. A similar, but rather poorly characterized example, was later described (53) in *Myxococcus*. Additional cases were described for strains of *Desulfovibrio desulfuricans* (43) and for the spirochete *Serpulina hyodysenteriae* (24), although in these the particles are larger, containing 13.5 kbp and 7.5 kbp, respectively, of DNA. The interesting question was raised (66) of whether capsduction evolved in the absence, or independently, of "true" (i.e. infectious, virally reproducing) bacteriophage. Which came first: capsduction and then bacteriophage, or bacteriophage and then transduction, followed, through loss of function, by capsduction?

Defective phage systems have been known for many years, although they have been usually detected (e.g. in many *Pseudomonas* and *Bacillus* strains) on the evidence of viral or subviral particles (35), recognized either morphologically by electron microscopy or through their bactericidal activity, rather than on the evidence of gene transfer experiments. In the case of the much studied PBSX defective prophage (approximately 33 kbp in size) present in *Bacillus subtilis* and in other species of this genus (1, 48, 54, 65), heterologous cell killing seems to have supplanted the gene transfer function, even though the particles contain a random sample of bacterial DNA (in fragments of 13 kbp), which, after extraction, is capable of transfection. Similar examples are those of PBLA (22) in *B. licheniformis* (producing particles containing 51 kbp host DNA fragments) and of PBND8 (57) in *B. natto* (particles with 8 kbp DNA fragments). In the absence of comparative studies of particle formation and its control, the difference between such defective lysogeny and capsduction may be only one of degree, depending on the extent to which viral functions are present.

An effort was made to test stringently to which extent VTA DNA was representative of *M. voltae* DNA. When VTA DNA was concentrated as much as feasible and then digested with *Alu*I (which statistically may be expected to cut *M. voltae* DNA approximately once every 360 bp), a new band of 0.9 kbp was observed (band φ, Fig. 7) which was not visible in similarly digested, bacterial DNA preparations. This band presumably represents a DNA sequence that is either amplified through replication relative to the rest of the bacterial genome, or preferentially packaged, in the course of the production of VTA particles. It remains an open question whether it is part of a presumptive prophage sequence.

Both PBSX of *B. subtilis* and GTA of *R. capsulata* are present in a majority of the strains classified within the respective species. The fact that VTA-like DNA could be demonstrated in three other isolates of *M. voltae*, strains C1, C2, and A3 (note however that C1 and C2 were isolated (62) from the same site), suggests that VTA may likewise be fairly typical of the *M. voltae* species. The negative results for attempted gene transfer from these three strains to PS would suggest the presence of exclusion mechanisms, probably DNA restriction and modification. Since chromosomal DNA of strain PS includes (64) some sequences that hybridize with the DNA of a plasmid present in strain been experimentally obtained (20), with VTA inclusions. These might then remain, at least for a time, associated with the cells that produced them. Such particles would obviously have significantly lower buoyant density than compact, phage-like particles. Vesicular structures derived from cell membranes and other surface material are known for several eubacteria: blebs in *Neisseria* (12), transformosomes in *Haemophilus* (31), predatory membrane vesicles in *Pseudomonas* (30) and others. Some of these structures are known to protect DNA from DNase and to participate in the transfer of DNA to other cells.

Cases of apparent reversion to wild type noted in the course of the isolation and purification of auxotrophic mutants from *M. voltae* (see Discussion in ref. 6) may well have been due to the then unsuspected presence of VTA.

From the point of view of microbiological technique, the setting free by filtration of a gene transfer agent largely bound to the cells is relevant to the interpretation of cases of gene transfer as due to conjugation: the control experiments in such cases ought to employ a culture filtrate and not simply (the filtrate of) a culture supernatant.

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LEGENDS TO FIGURES

FIG. 1. Histidine-independent colonies resulting from a constant amount of filtrate and variable recipient bacteria input. Donor strain: PS-13. Recipient strain: PS-3. Standard pour plate technique (10 ml agar per plate).

FIG. 2. VTA-mediated transfer of *bes* to BES-sensitive bacteria. Recipient bacteria: PS-1, about 1.6×10^6 per plate. VTA from strain PS-2. Each point represents the colonies of one plate. A 5 ml agar overlay with BES was added after 2 days incubation, establishing upon diffusion a 1.1 mM BES concentration. Two control plates without VTA gave 12 and 18 resistant colonies: the data as plotted are corrected for this background. The dashed line represents the best fit for direct proportionality.

FIG. 3. VTA cumulatively recovered in successive extractions (washings) of a culture of *M. voltae*, strain PS-2. Recipient strain: PS-3. Selection: histidine independence. F_0, filtered culture supernatant; F_1 through F_5, filtrates of supernatants obtained in five washing cycles; F_p, filtrate of the last resuspended pellet. The culture, 52 ml at 2×10^8 cells per ml, was resuspended each time in 10 ml 0.3 M NaCl, 0.015 M Na citrate. Total *his*$^+$ VTA recovered: 4.3×10^6.

FIG. 4. Relationship between VTA titer and concentration of UV absorbing material in the filtrates of Fig. 3 (F_0 excluded). The mean A_{280}/A_{260} ratio was 0.55 (range 0.51 - 0.59).

FIG. 5. Isopycnic banding of *his*$^+$ VTA and two bacteriophage markers in a CsCl density gradient (Spinco rotor SW50.1, 27 hours, 41.5 krpm, 8°C). The expected buoyant densities for P2 *lg del1 del2* and for ΦX174 are, respectively, 1.42 (3, 7) and 1.40 (50). The VTA sample was prepared from strain PS-2 by the PEG-bag method, hence high concentrations of NaCl (0.5 M final in the centrifuge tube), BSA (tentatively 8 mg/ml), and added glycerol (0.6 M), were present in addition to cell components set free by filtration. The solid line for density is from refractometer readings for a control centrifuge tube containing only CsCl in pure water. The dashed line gives the gravimetrically determined densities for reconstructed solutions of corresponding concentrations of CsCl, plus NaCl and glycerol. Comparison of refractometer readings for such reconstructed solutions with those taken over the experiment gradient (data not shown) suggests that BSA and/or cell proteins were beginning to band in the upper part of the gradient. Recovery of activities: 1% of input for VTA, 65% for ΦX174, and >28% for P2.

FIG. 6. Agarose gel electrophoresis of phenol extracts showing the VTA DNA band. Size markers (lane 1 in A, B, C, D and E, lane 2 in F) are fragments of a *Hin*dIII digest of phage λ DNA (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6 kbp).

A, lane 2: DNA from whole bacteria, strain PS-6. B, lane 2: extract from partially purified (see under Results) VTA, strain PS-6. C, lane 2: extract from partially purified (procedure (a) in Materials and Methods) VTA, strain PS-13. D, lane 2: extract from a concentrated filtrate (PEG-bag method), strain PS-2. The sample applied was the equivalent of about 8 ml of culture. E, lane 2: DNA from a "short cut" preparation (see

Material and Methods), strain PS-13. F, lane 1: VTA DNA (strain PS-1) exposed to 85°C for 3 min; lane 3: unexposed control VTA DNA; lane 4: *Hin*dIII digest of phage λ DNA exposed to 85°C for 3 min. G, lane 1: unexposed control VTA DNA (strain PS-1); lanes 2 through 5: VTA DNA exposed for 1 min to 78°, 80°, 82°, and 84°C, respectively. Size markers for G (part of the same gel slab) are seen in Figure 7 C, lane 2; the magnification is different in the two figures, however. Note that in G the agarose concentration was higher and the voltage applied lower than in A through F.

FIG. 7. Fragment size distribution in agarose gel electrophoresis of *M. voltae* DNA and concentrated VTA DNA after exhaustive digestion with restriction endonuclease *Alu*I. Size markers as in Fig. 6. Electrophoresis conditions as in Fig. 6, G.

A, lanes 1, 2 and 3: decreasing amounts (in 4:2:1 ratio) of *M. voltae* DNA; lane 4: size markers; lane 5: concentrated VTA DNA. B, lane 1: VTA DNA; lane 2: size markers; lane 3: undigested VTA DNA (amount applied is very roughly 1/100 of that in lane 1); lane 4: *M. voltae* DNA. C, lane 1: concentrated VTA DNA; lane 2: size markers; lane 3: same as lane 1, but exposed to RNase before application to gel.

Table 1. Derivatives of *Methanococcus voltae* PS

Collection no.	DSM no.	Genotype	Origin
PS-1	1537	Wild type	(see ref. 61)
PS-2		*bes*	(6)
PS-3	4254	*his*	(6)
PS-5		*his mtp*	(6)
PS-6	4310	*bes pur cbl*	(6)
PS-9		*bes cbl*	(6)
PS-12			Subclone of PS-1, (6)
PS-13		*bes pur cbl x?*	From PS-6, see BES resistance, in Materials and Methods
PS-15		*bes pur*	From experiment J2 in Table 2

Abbreviations: *cbl*, *his*, *pur*: requirement for vitamin B12, histidine or purine, respectively; *bes*, *mtp*: resistance to 2-Bromoethanesulfonate or 5-Methyl-tryptophan, respectively; DSM: Deutsche Sammlung von Mikroorganismen, Braunschweig.

FACSIMILE OF "CAMERA READY"
Tables 2 & 3

TABLE 2. Transfer of prototrophy to *M. voltae* auxotrophic mutants by culture filtrates

[1] Expt[a]	[2] Recipient strain[b]	[3] Donor strain[b]	[4] Preparation and treatment of filtrate[c]	[5] Volume of filtrate per plate[d]	[6] Selection[e]	[7] Number of colonies per plate[f]
A1	PS-5 (0.55)	None		0	His^+	1
A2		PS-6 (0.68)	A, ST	1; 10	His^+	~3,140; ~30,000
A3			SUP, A	1	His^+	105
A4	PS-6 (0.68)	None		0	Pur^+	0
A5		PS-5 (0.55)	A, ST	1; 10	Pur^+	188; ~1,980
B1	PS-6	None		0	Pur^+	1
B2		PS-12 (0.80)	A	2	Pur^+	~1,000
B3			As above, heated 2 min at ~89°C	2	Pur^+	0
C1	PS-3 (0.56)	None		0	His^+	0
C2		PS-6 (0.39)	A	1; 10[g]	His^+	66, 98; 488
C3			As above, diluted 1:20 in WM medium	1[g]	His^+	6, 4
C4			Same as in C2, treated with DNase[h] at 25 μg/ml for 10 min	1[g]	His^+	61
C5			Same as above, DNase at 5 μg/ml	10	His^+	354
D1	PS-6 (0.63)	None		0	Pur^+	0
D2		PS-1 (0.60)	U	3	Pur^+	157
D3		PS-12 (0.83)	U	3	Pur^+	141
E1	PS-6 (0.52)	None		0	Pur^+	0
E2	PS-13 (0.58)	None		0	Pur^+	0
E3	PS-6	PS-12 (0.72)	A, ST	1	Pur^+	1,030
E4	PS-13		As above	1	Pur^+	760
E5	PS-6		SUP, A, ST	1	Pur^+	13
E6	PS-13		As above	1	Pur^+	6
E7	PS-6		PELLET (suspension in WM), A, ST	1	Pur^+	549
E8	PS-13		As above	1	Pur^+	400
E9	PS-6		Same as in E3	1	Pur^+ (Cbl^+)	522[i]
E10			As above	1	(Cbl^+)	883[i,j]
E11			Same as above, exposed to 90°C for 2 min	1	Pur^+ (Cbl^+)	0[i]
E12			As above	1	(Cbl^+)	57[i,j]
F1	PS-3 (0.66)[k]	PS-6	A, first ml of filtrate	20 μl	His^+	19
F2			Same, next 14 ml of filtrate	20 μl	His^+	805
F3			Last ml of filtrate	20 μl	His^+	137
G1	PS-3	None		0	His^+	0
G2		PS-15	A, ST	10 μl	His^+	549, 468
G3			Same, diluted 1:100 in WM medium	1 ml	His^+	472, 439
G4			Same, diluted 1:100 in distilled water	1 ml	His^+	398, 524
H1	PS-6 (0.48)	None		0	Pur^+	0
H2		PS-3	A	20 μl	Pur^+	100
H3			SUP, A	20 μl	Pur^+	4
I1	PS-3	None		0	His^+	0
I2		PS-6 (~0.3)	A	10 μl; 200 μl	His^+	103; ~1,340
I3			SUP, A	10 μl; 200 μl	His^+	2; 17
I4			PELLET (in 0.6 M NaCl, 0.06 M Na-citrate, kept 5 hours in ice) then SUP, A	10 μl; 200 μl	His^+	57; 1,089
J1	PS-6[k,l]	None		0	Cbl^+	13; 4; 10[l]
J2		PS-12 (0.34)	A	1	Cbl^+	75; 40; 42[l]
J3			Same, heated 5 min at nearly 100°C	1	Cbl^+	13; 7; 14[l]
J4	PS-3 (0.36)[k]		As above	1	His^+	0
J5			Same as in J2	1	His^+	20
K1	PS-3	PS-13	U	10 μl	His^+	1,201
K2			Same, sucked in and out 30 times with syringe through gauge 22, 1 inch needle	10 μl	His^+	623

[a] Procedures for VTA assay as described in Materials and Methods. Unless noted otherwise, the amount of recipient bacteria per plate was one syringe drop of a fresh, visibly turbid, undiluted culture.

[b] The number in parentheses is the optical density of the culture used. In some cases it was not recorded.

[c] Filters used: A, Acrodisc; U, UNIFLO (see Materials and Methods). Filtrates were prepared anaerobically and used shortly after. ST, filtrate was checked for sterility by inoculating appropriately supplemented WM. At times the culture was first spun at low speed to remove the bacteria, and the supernatant only was filtered and used (SUP), or the pellet was resuspended in the same volume of fresh medium and filtered (PELLET), or the new suspension was centrifuged as above, and this second supernatant filtered and used (PELLET then SUP)

[d] Volume given as number of syringe drops , unless otherwise specified. One drop may be assumed to be on the average 14 µl. The composition of the medium and several other factors will obviously affect drop volume.

[e] Selection medium: WM agar. Supplements: cyanocobalamin in A, B, D, E1 through E8, H; hypoxanthine in E10, E12, J1, J2, J3.

[f] Duplicate plates separated by comma and space.

[g] Drops of recipient culture and filtrate were mixed 20 to 40 min before addition of melted soft agar medium.

[h] Sigma crude DN-25. Under exactly the same conditions the enzyme was shown to be fully active on a *M. voltae* DNA preparation.

[i] See text.

[j] In addition to the colonies recorded here, very large numbers (uncountable) of very tiny colonies were present in this plate. These were presumably derived from untransformed recipient cells trying to grow in the absence of cyanocobalamin. They were absent in E9 and E11, where the much tighter requirement for purine did not allow any residual growth.

[k] Two drops of recipient culture were used.

[l] Three cultures of the recipient, grown in 1/2, 1/20, and <1/200 of the standard (100 nM) cyanocobalamin concentration, were used in parallel. Their optical densities were, respectively, 0.33, 0.31, and 0.20. Colony numbers obtained from each are given in column 7, in the same sequence. From the first plate in J2 one of the larger colonies (thus presumably not a revertant) was isolated as strain PS-15.

TABLE 3. Decay rates of VTA (as *his*⁺ transfer) under various storage conditions[a]

Prepn	Gas phase	Temp	Mo[b]	Medium[c]	H_{mo} (n)	H_0
1	anox[d]	-70°C	31.5	WM PEG 10%	0.13 (4)	
2a	air	0°C	0.8	WM	6.9 (2)	1.0
2b	air	0°C	0.8	WM BSA 0.05%	5.0 (2)	0.4
3a	anox[d]	+5°C	4.1	0.75M NaCl	0.64 (10) (0.5 - 0.8)[e]	2.1
3b	air	+5°C	0.4	As above	0.73 (1)	1.4
4	anox[d]	+5°C	4.4	0.3M NaCl	1.1 (8) (1.0 - 1.3)[e]	
5a	air	0°C	3.0	0.3M NaCl BSA 0.1%	0.82 (3) (0.5 - 1.4)[f]	1.4
5b	air	0°C	3.0	~0.3M NaCl and $MgCl_2$[g] BSA 0.1%	0.65 (8) (0.3 - 1.3)[f]	1.5
6a	air	0°C	3.0	0.3M NaCl BSA 0.1% Glycerol 10%	1.9 (5) (1.6 - 2.2)[f]	-0.2
6b	air	-70°C	4.3	As above	0.62 (4) (0.1 - 1.8)[f]	0.6
7a[h]	air	0°C	4.3	0.3M NaCl BSA ≥0.1%	0.52 (4) (0.1 - 1.8)[f]	
7b[h]	air	0°C	5.8	As above	0.33 (5) (0.0 - 0.6)[f]	
8	air	0°C	1.1	0.3M NaCl Glycerol 20%	1.9 (4) (0.9 - 2.9)[f]	
9a	air	0°C	1.2	0.3M NaCl	2.4 (2)	2.9
9b	air	0°C	2.7	0.3M NaCl BSA 0.1%	1.6 (3)	0.7
9c	air	0°C	2.7	0.3M NaCl BSA 1.%	0.75 (3)	1.1
10a[i]	air	-80°C	6.9	0.3<M<0.8 NaCl Glycerol 10% BSA present	0.56 (3)	0.6
10b[i]	air	-80°C	6.9	NaCl and BSA: lower concn than above Glycerol 10%	0.59 (3)	1.4
10c[i]	air	-80°C	6.9	As above	0.22 (3)	1.2
11[j]	air	-18°C	2.3	~0.2M NaCl PEG 10%	1.1 (1)	1.2

[a] H_{mo} (n) and H_0 are defined in Materials and Methods.
[b] Total duration of storage in months over which titrations were made.
[c] Filterable debris from bacteria lysed in the course of filtration might affect VTA stability. WM (the standard culture medium) refers here to whole culture filtrates. Bacteria were also present at filtration when preparations 10 and 11 were made by the PEG-bag method. For all other preparations in the Table, bacteria had been removed by centrifugation before filtration (see Material and Methods). Where NaCl is indicated (with the exception of preparation 5), either citrate (5 to 15 mM, pH 6.6) or Tris (20 mM, pH 7.5 to 7.9, with up to 2 mM EGTA) were also present. There was no evidence of significant differences in VTA stability with these additions.
[d] Gas from the anaerobic hood: N_2 with small amounts of CO_2, H_2 and probably traces of H_2S.
[e] In parentheses, H_{mo} ± standard error.
[f] In parentheses, range of H_{mo} values.
[g] Various concentrations of $MgCl_2$ (between 14 and 41 mM) at constant ionic strength. Pooled data.
[h] A 1:6 dilution of a VTA preparation concentrated 200-fold in a Centricon 500 centrifugal concentrator with BSA present.
[i] Undiluted VTA preparations (PEG-bag method). The concentrations of the components of the suspension medium are thus not precisely known. Preparation 10a is the first sample collected from the dialysis bag after concentration; 10b and 10c are samples of the second distilled water rinse from the same bag. Titers (as *his*⁺ transfer) at collection were 6×10^6/ml in 10a and 5×10^6/ml in 10b and 10c.
[j] A 1:21 dilution of the first distilled water rinse in the PEG-bag preparation method.

Fig. 1

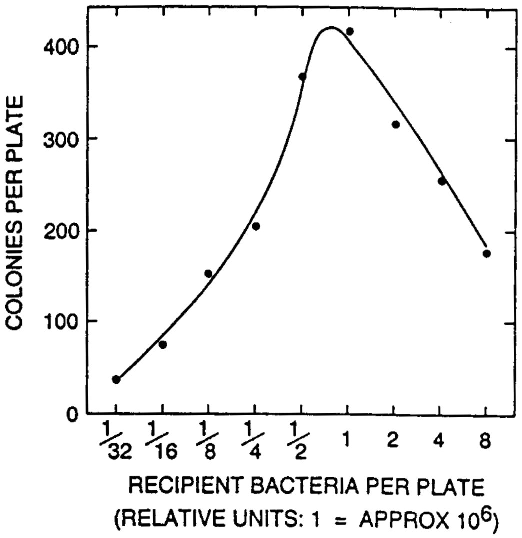


Fig. 2

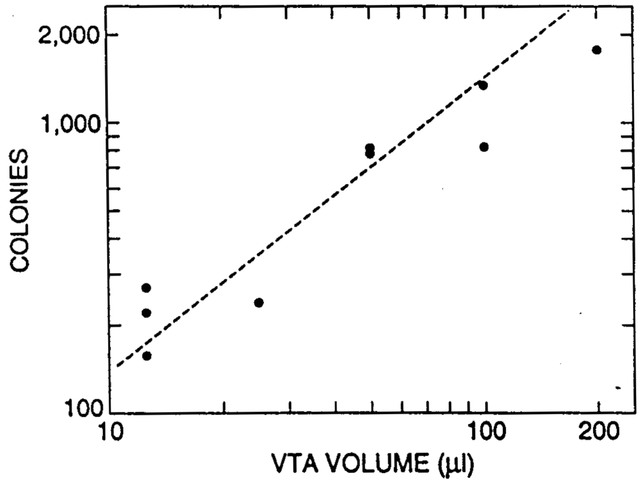


Fig. 3

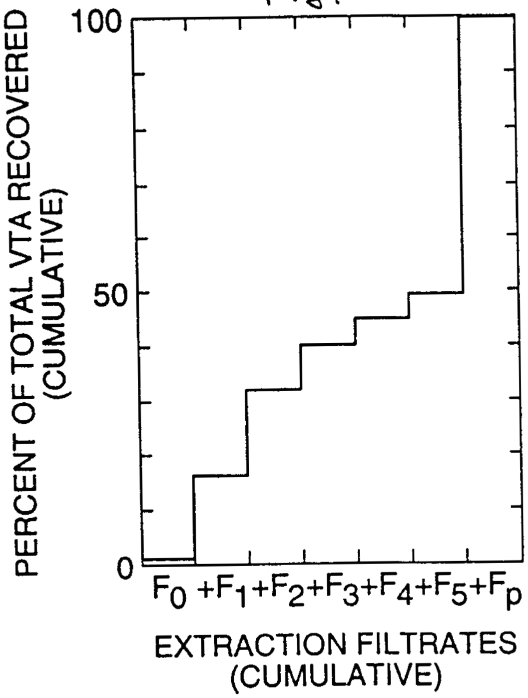
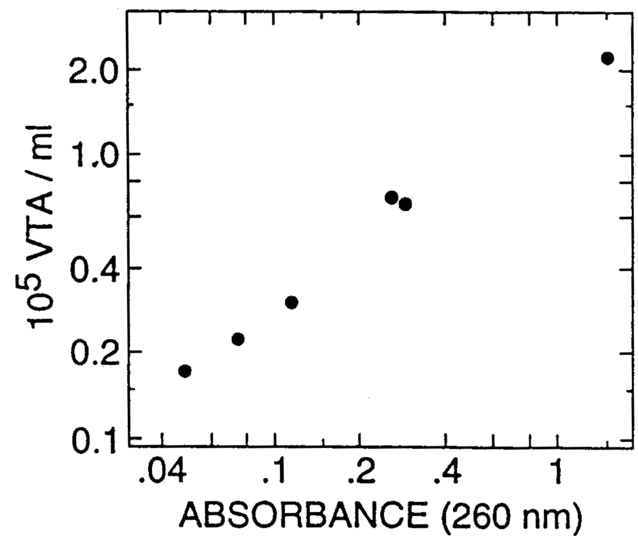
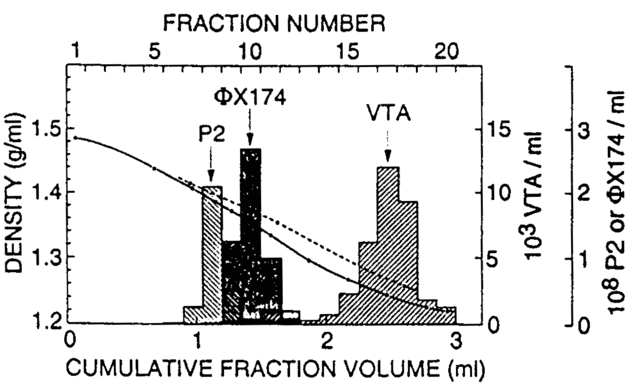


Fig. 4





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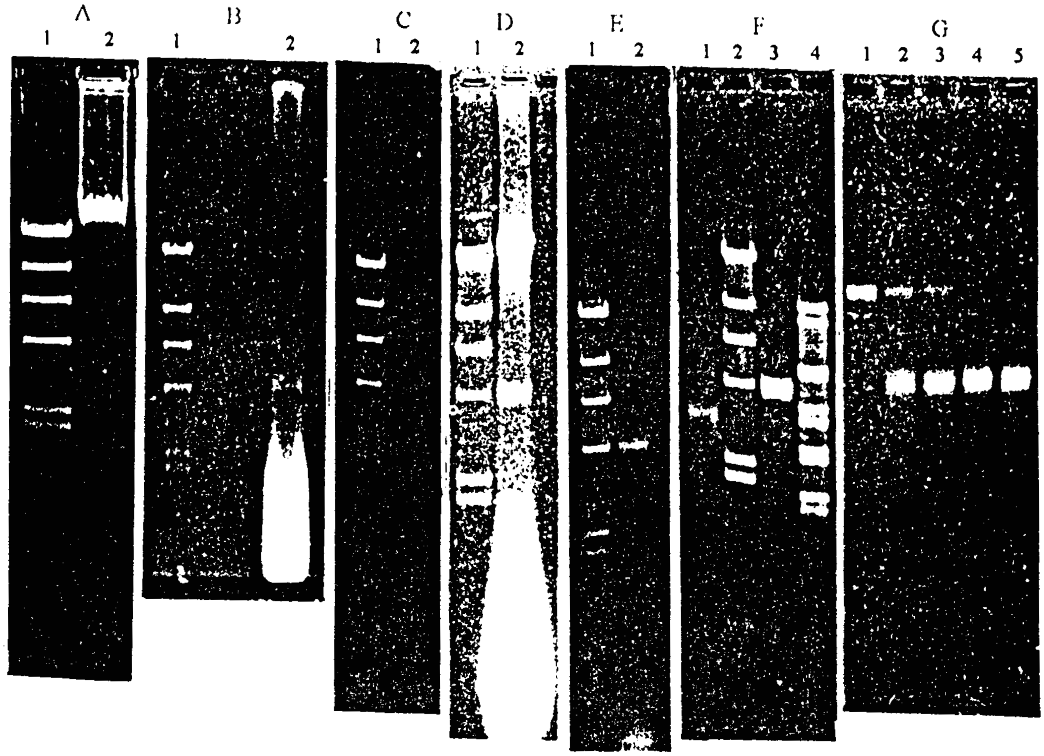

Fig. 6

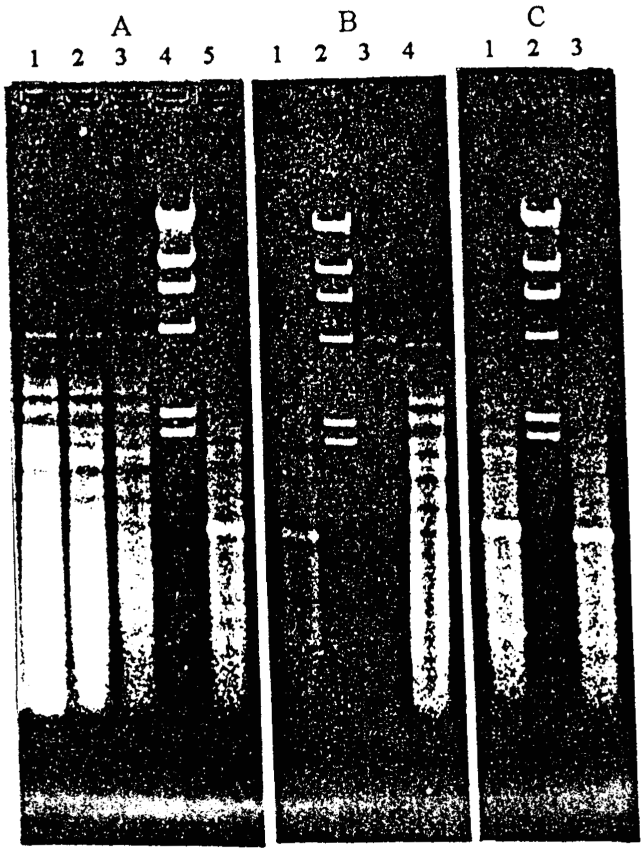

Fig. 7